

Aerosol immunization with NYVAC and MVA vectored vaccines is safe, simple, and immunogenic

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Each year, approximately five million people die worldwide from putatively vaccine-preventable mucosally transmitted diseases. With respect to mass vaccination campaigns, one strategy to cope with this formidable challenge is aerosol vaccine delivery, which offers potential safety, logistical, and cost-saving advantages over traditional vaccination routes. Additionally, aerosol vaccination may elicit pivotal mucosal immune responses that could contain or eliminate mucosally transmitted pathogens in a preventative or therapeutic vaccine context. In this current preclinical non-human primate investigation, we demonstrate the feasibility of aerosol vaccination with the recombinant poxvirus-based vaccine vectors NYVAC and MVA. Real-time *in vivo* scintigraphy experiments with radiolabeled, aerosol-administered NYVAC-C (Clade C, HIV-1 vaccine) and MVA-HPV vaccines revealed consistent mucosal delivery to the respiratory tract. Furthermore, aerosol delivery of the vaccines was safe, inducing no vaccine-associated pathology, in particular in the brain and lungs, and was immunogenic. Administration of a DNA-C/NYVAC-C prime/boost regime resulted in both systemic and anal-genital HIV-specific immune responses that were still detectable 5 months after immunization. Thus, aerosol vaccination with NYVAC and MVA vectored vaccines constitutes a tool for large-scale vaccine efforts against mucosally transmitted pathogens.

MVA HPV | non-human primate | NYVAC HIV | preclinical study | aerosol vaccine assessment

Despite the unprecedented medical success of vaccination, traditional application routes, i.e., intramuscular or s.c., are not without their limitations. In the context of mass vaccination campaigns in developing countries, obstacles exist including high costs and safety considerations (1). Additionally, in the case of some mucosally transmitted pathogens, such as HIV and HPV, traditional vaccination routes may not induce befitting mucosal immune responses needed to afford prophylactic protection or therapeutic effect (2). Thus, exploring alternative vaccination routes that may overcome these obstacles are warranted. Aerosol immunization is a prime example of such an alternative. It offers many advantages including ease and speed of application by nonmedical personnel, noninvasiveness resulting in greater social acceptance, reduced risk of cross-contamination of blood-born infectious agents, diminished medical waste, and potentially lower costs (3, 4).

NYVAC and MVA are two highly attenuated recombinant poxvirus-based smallpox vaccines. Both derived from vaccinia virus strains, NYVAC and MVA were rendered replication deficient in mammalian cells by removing >10% of their genomes either by selective gene deletion or serial passage in chicken eggs (5, 6). These resulting poxvirus-based vaccines have proven to be extremely safe and well supported in human volunteers. In the literature, multiple human clinical trials using these vectors have been reported, and MVA alone has been administered to >100,000 volunteers. To date, no serious adverse events have been described. Given this impressive safety profile, in conjunction with each vector's capacity to carry up to 10 kb of insert DNA, NYVAC and MVA have evolved into vaccine vectors, capable of evoking potent cellular and humoral immune responses against their insert immunogens, especially when used in a DNA-prime/poxvirus-boost immunization schedule (7, 8).

The aim of this current collaborative preclinical study was to assess aerosol administration of the recombinant poxvirus-based vaccine vectors NYVAC and MVA by using a non-human primate model. The scope of this work included characterization of appropriate aerosol administration conditions, deposition of the vaccines at mucosal surfaces upon delivery, and, most importantly, safety, in particular with regard to the brain and respiratory tract. Furthermore, the immunogenicity of aerosol-administered NYVAC was assessed by looking at cellular and humoral immune responses. To our knowledge, it is the first investigation of this kind that demonstrates the safety and immunogenicity of aerosol immunization with the recombinant poxvirus-based vaccine vectors NYVAC and MVA.

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ations were seen, such as slight lymphoid cell infiltrations in the sinus and pharynx (Fig. 2*B*). Each of these findings was confined to single, but different, animals. Moreover, the tissues from these organs appeared, overall, to be healthy, retaining delicate microstructures such as cilia in the sinuses and trachea (Fig. 2*C* and SI Fig. 8*C–E*).

The fine lung architecture of the aerosol-vaccinated animals was found entirely intact and normal (Fig. 2*D* and *E*). No signs of gross pathology were seen. The only vaccine-associated changes were found in the MVA-HPV administered animals, where minimal to slight bronchiolar cuffing was observed (SI Fig. 8*F*). These changes were all minor in nature, similar to those seen during a common cold. Common to all animals were minimal, multifocal pigment deposits in the pulmonary macrophages. These deposits resemble refractile spicules indicating the presence of mites and hence were considered vaccine-unrelated (data not shown) (9). In one NYVAC-C-administered animal, multiple, moderate foreign-material granuloma were identified in the lungs. The material was not of vaccine origin and was most likely bits of monkey chow. An identical finding has been reported in a previous study (10).

In other organs, namely the liver and kidneys, minimal to slight microscopic findings were observed (SI Table 1). These changes, however, were considered to be incidental and part of the normal background commonly observed in outdoor-bred rhesus macaque monkeys. A severe hepatic granuloma was found in one animal (F3, MVA-HPV). Considering that the animal had elevated alanine transaminase (ALT) levels at the time of the study inclusion and that the chronic nature and incidence of histological changes did not indicate a relationship with the vaccination, the observed liver alterations were not considered by independent pathologists to be caused by vaccine administration. Of further note was a minimal lymphocytic infiltration found in an eye of one animal. This finding was restricted to a single monkey. Overall, this safety analysis demonstrated that there was no vaccine-induced pathology and that aerosol administration of NYVAC-C and MVA-HPV is safe.

Immunogenicity. Given the safety of aerosol-administered poxvirus-based recombinant vaccines, a further study was undertaken to investigate immunogenicity. Two cohorts of rhesus macaque monkeys, an aerosol group ($n = 10$) and an intramuscular (i.m.) control group ($n = 5$), were immunized by using a prime/boost schedule. All animals received 4.2 mg DNA-C (DNA vaccine) i.m. at weeks 0 and 4, and subsequently received $1 \times 10^{7.7}$ pfu NYVAC-C at weeks 20 and 24, because either an aerosol or i.m. DNA-C and NYVAC-C contain identical codon-optimized Clade C HIV-1 genes encoding gp120 and a Gag-Pol-Nef fusion protein (SI Fig. 9) (11). The immunization schedule was adapted from a previous DNA-C/NYVAC-C i.m. study (P.M., S.B.-J., N.B., P. van Haaften, I. Baak, I. Niewenhuis, S. Heidari, H. Wolf, J. Frachette, A. Harari, G. Pantaleo, K. Bieler, N. Sheppard, J. Liljestrom, R. Wagner, J.L.H., unpublished data) to allow comparisons. The identical vaccines, vaccine quantities, and immunization schedule (i.m. application) have been recently used in a EuroVacc Phase I clinical study (12).

Humoral Immune Responses. Sera samples were taken to measure humoral immune responses against the HIV envelope protein and the vaccinia vaccine vector backbone. An IgG HIV-specific response (week 24) was detected after DNA-C immunization (Fig. 3*A*). This response remained stable and was further boosted in both macaque cohorts upon NYVAC-C administration. The i.m. response was initially slightly higher in magnitude but within 1 month approached that of the aerosol immunized group. By study-week 48, HIV-specific IgG responses in both groups remained only slightly higher than assay background. No HIV-specific IgA sera responses were detected (data not shown).

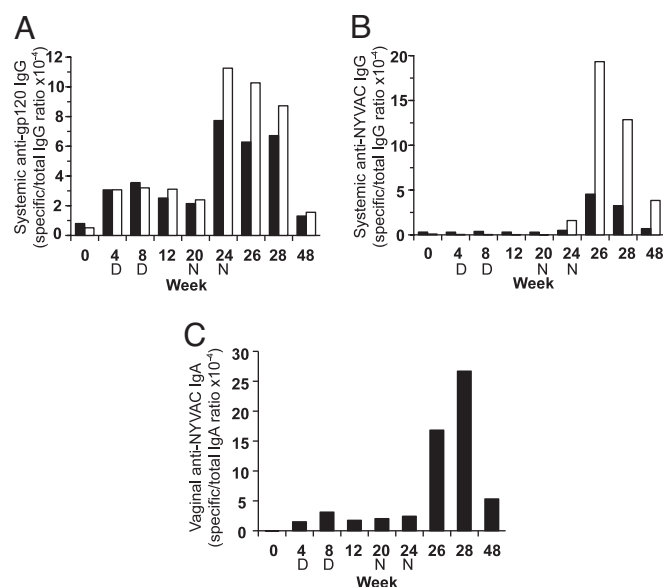


Fig. 3. Humoral immune responses induced by aerosol vaccination. (A) Systemic anti-gp120 IgG response. (B) Systemic anti-NYVAC IgG response. (C) Vaginal anti-NYVAC IgA response. Vaccination times are indicated by “D” and “N” for the DNA-C and NYVAC-C vaccines, respectively. Closed bars and open bars represent aerosol and i.m.-immunized animals, respectively.

Vector-specific IgG responses were first detected at study-week 24, 1 month after the first NYVAC-C immunization and were significantly higher in the i.m. vaccinated group (Fig. 3*B*). No HIV-specific IgA antibodies were detected in the nasal, vaginal, and rectal secretions. However, NYVAC-specific IgAs were found in three of five female animals vaccinated with the NYVAC-C aerosol (Fig. 3*C*). No consistent vaginal IgA response was measured in the female animal immunized i.m., and no consistent nasal or rectal NYVAC-specific IgA responses were observed in either cohort (data not shown).

Cellular Immune Responses. Aerosol delivery of NYVAC-C elicited strong systemic cellular immune responses (Fig. 4) mirroring those induced by i.m. vaccination. IFN γ and IL-2 HIV-specific responses, as measured in PBMCs by ELISPOT analysis, were already detectable after DNA-C priming (Fig. 4). These responses peaked at study-week 24, after NYVAC-C administration, and remained robust until study’s end 6 months later.

In contrast to IFN γ and IL-2, HIV-specific IL-4 cellular responses were more difficult to induce and maintain. No significant responses were seen after DNA-C immunization. After NYVAC-C administration, both as an aerosol and i.m., an intense IL-4 response was observed. This response, however, was transient in nature. By week 48, IL-4 responses had returned to background levels in the aerosol-immunized group and were markedly decreased in the i.m.-vaccinated group.

To investigate the breadth of the cellular immune responses induced by aerosol administration of NYVAC-C, eight distinct pools (two env, two gag, three pol, and one nef) of overlapping 15-mer peptides spanning the entire HIV immunogen were used to screen peripheral blood mononuclear cells (PBMCs) (SI Fig. 9). After DNA-C vaccination, few IFN γ and IL-4 responders were seen. For IL-2, responses were focused on the two env peptide pools (Fig. 5). After aerosol immunization with NYVAC-C, broad cellular immune responses were evoked. Responders for all three cytokines for all eight HIV peptide pools were observed. Immune responses directed against the two env peptide pools were again the most prevalent. By study-week 48, the number of IFN γ responders for all peptide pools remained

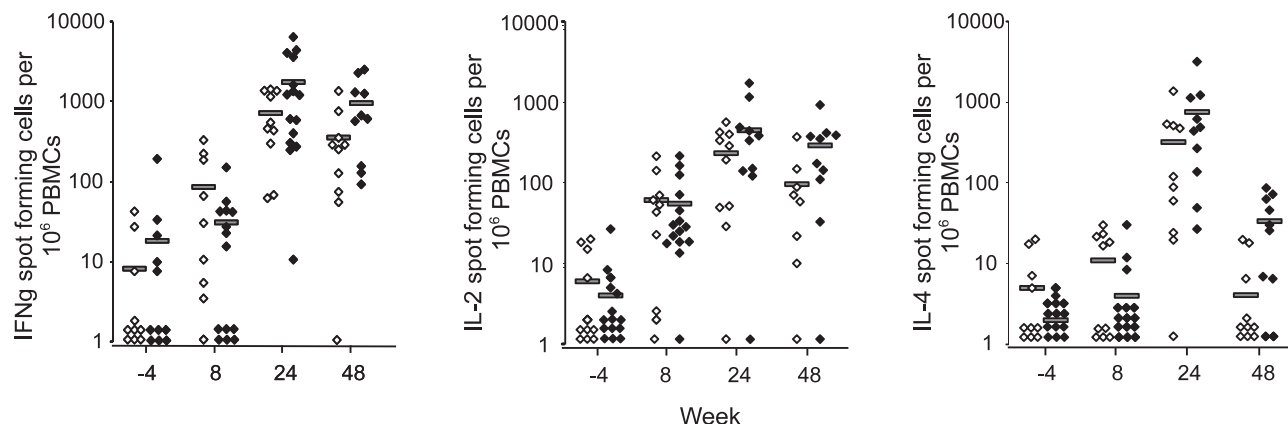


Fig. 4. Cellular immune responses induced by aerosol vaccination. Shown are induced HIV-specific IFN γ (Left), IL-2 (Center), and IL-4 (Right) systemic cellular immune responses in the two vaccinated cohorts as measured by EILISPOT analysis. The average mean response is illustrated by a gray bar. The responses are the sums of eight HIV peptide pools.

broad and comparable to study-week 24. The number of IL-2 responders remained high for the peptide pools gag 1, env 1, and env 2. Essentially no IL-4 responders were seen at the end of the study. With the exception of IL-2 and IL-4 at study-week 48, the number of responders in the i.m.-immunized group was com-

parable to the aerosol immunized group. Furthermore, no significant differences were seen in the average number of peptide pool responses per animal between the two groups at each time point (SI Fig. 10). Collectively, these results demonstrate that aerosol administration of a poxvirus-based vaccine can induce robust systemic cellular immune responses directed against its insert immunogens. Furthermore, these responses are broad and, depending on the cytokine response, long-lasting in nature.

To detect the presence of mucosal cellular immune responses, biopsies were taken for analysis from vaginal and rectal tissues at weeks 4 and 48. Lymphocytes, isolated from 5-mm snip samples, were expanded in an antigen-independent manner (ref. 13; also see SI Methods). Week-4 and -48 cultures, from eight animals, six aerosol- and two i.m.-immunized, were established. Intracellular cytokine staining (ICS) FACS analysis, monitoring for the production of IFN γ , IL-2, and IL-4 in response to stimulation with either env or gag peptide pools, revealed no HIV-specific responses in all week-4 samples tested. However, at study-week 48, both env- and gag-specific IFN γ , IL-2, and IL-4 responses were seen (SI Fig. 11 and SI Table 2). Intramuscular-immunized animals exhibited mucosal immune responses as well, as seen in a previous intranasal immunization study (14). Because of variable sample sizes, sample compositions, and extents of cell expansions, significant intraanimal quantifications were not possible. No differences in the total number of lymphocytes in the mucosal tissues of both groups were observed before and after vaccination (immunohistochemistry analysis, data not shown).

Discussion

We show here that aerosol vaccination with recombinant poxvirus-based vaccine vectors is safe and evokes robust, long-lasting immune responses against the vaccine insert immunogens. The results from this non-human primate preclinical investigation demonstrate the feasibility of aerosol vaccination by using NYVAC and MVA in future human clinical trials.

During our safety evaluation, no vaccine-associated pathology was observed in the brains of the six animals examined. These results are in accordance with previous experiments performed in mice (5, 15). Furthermore, indirect evidence of neural safety is illustrated by the fact that during the 6-month period, no erratic behavior or physiological changes were observed after aerosol administration of NYVAC-C. Similar findings have been reported for immunocompromised rhesus macaques given even higher doses of MVA intranasally, i.m., and intradermally si-

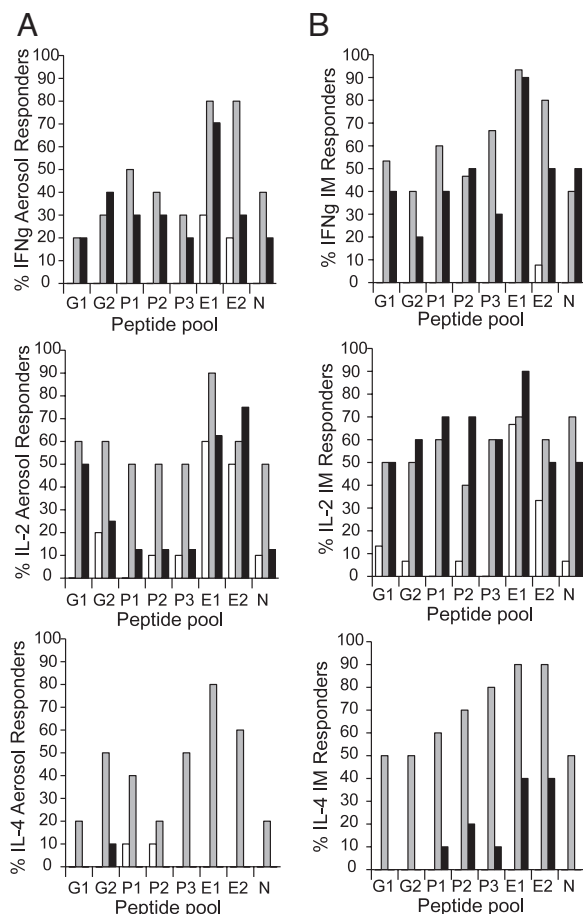


Fig. 5. Broad-breadth cellular immune responses induced by aerosol vaccination. (A) Percentage of aerosol responders to individual peptide pools. (B) Percentage of i.m. responders to individual peptide pools. Week-8, -24, and -48 responses are shown as white, gray, and black bars, respectively. G, Gag; P, Pol; E, Env; N, Nef.

multaneously (16). A careful pathological evaluation of the brain, as performed in the current study, however, was not described.

A concern raised during the deposition/safety study was ocular vaccine exposure. One of the two animals killed for direct scintigraphy exhibited low amounts of radioactivity in the eye samples not detectable by *in vivo* real-time measurements. Moreover, one of the six animals examined for histological changes had a slight ocular lymphocytic infiltration. Given the small number of animals analyzed, it is difficult to conclude whether such a putative vaccine exposure to the eyes would pose a risk to vaccinees. Nevertheless, future human clinical trials should be conducted with a mouthpiece instead of a face mask.

Within the respiratory tract, no gross pathology was detected. Slight perivascular cussing in the lungs was common to the MVA-HPV aerosol-vaccinated animals. This occurrence, although vaccine-associated, was microscopic in nature and did not constitute tissue pathology. It most likely represented reversible histological changes involved with vaccine uptake and initiating immune responses.

An important facet of the immunogenicity trial were the broad-breadth, durable immune responses induced by aerosol vaccination despite limited vaccine delivery, $7.42\% \pm 1.47\%$ total deposition as measured during the deposition study. The respiratory tract is in constant contact with a microbial burden second only to the digestive tract. To cope with this microbial load, it is equipped with, amongst others, a dense mesh work of antigen-presenting cells throughout the nasal cavity, trachea, and lungs to initiate appropriate adaptive immune responses (17, 18). Furthermore, specialized mucosal-associated lymphoid tissues, such as the Waldeyer's ring at the back of the throat, actively sample lumen antigens to initiate systemic and mucosal immune responses (19). Evident from the real-time *in vivo* scintigraphy measurements was an even distribution of vaccine over the entire respiratory tract, with a distinct accumulation in the area of the Waldeyer's ring. Thus, despite the low amount of delivered vaccine, because of the efficient antigen-uptake capacities of the mucosal tissues, potent immune responses were generated.

Except for the cellular systemic IL-4 response, all aerosol vaccine-induced immune responses were either equal to or slightly less than those induced by i.m. immunization. It should be kept in mind that these differences between the two vaccination routes may well be specific to the animal model used. Because of their small size, physiology, and shallow tidal breathing patterns, rhesus macaques have a lower capacity to take up aerosols in the respiratory tract (20). This lower capacity is illustrated by the fact that the same nebulizer used in human studies has delivered 10–30% of the administered product to the lungs alone, as compared with 1.5% and 3.5% for NYVAC-C and MVA-HPV, respectively (21–23). Further evidence illustrating this point comes from aerosol administration of measles vaccines in infants 12 months and younger who, from the point of view of aerosol vaccination, are physiologically comparable to small non-human primates. Contrary to older children and adults, these infants developed inferior immune responses after aerosol vaccination compared with i.m. administration (24, 25). These data, taken together, suggest that aerosol-induced NYVAC and MVA vaccine immune responses in humans may be at least as potent as those induced by i.m. vaccination.

Dampening of immune responses induced by poxvirus-based vaccines may be a concern because of preexisting immunity to vaccinia. The extent to which this will play a role for these vaccines remains to be established considering that smallpox vaccination was discontinued in the early 1980s and HIV infection spreads predominantly in the young populations in developing countries. Additionally, in a EuroVacc Phase I clinical trial (15), robust vaccine-induced T cell responses were

observed in vaccinees regardless of preexisting pox immunity after the identical DNA-C prime/NYVAC-C boost vaccination regimen. Only a slight trend of possibly blunted immune responses was observed (A. Harari and J. Pantaleo, personal communication). A further consideration is evidence in the literature that mucosally applied vaccines can overcome preexisting vector immunity (26, 27). Given these various facets, additional investigations are required.

Mucosal immune responses may be needed to prevent or control infectious agents, such as HIV or HPV, that enter the body through and/or replicate in mucosal tissues. In response to NYVAC-C aerosol vaccination, animals in the current study developed distal anal-genital cellular and humoral immune responses, which were measurable at the study's end. The vector-specific IgA antibodies, detected in vaginal secretions, were in accordance with those demonstrated in women nasally immunized with the potent mucosal adjuvant cholera toxin (28). However, in the current study, no mucosal IgA responses directed against HIV gp120 were detected. At the moment, the reason for this observation is unclear and may be related to variables such as T cell-independent Ab responses to repetitive viral antigens, stimulatory effects of innate immune responses triggered by the vaccine vector, or delayed expression of HIV immunogens upon vaccination. Interestingly, a recent report has demonstrated that poxvirus-based vaccines are themselves adjuvants for coadministered proteins (29). Therefore, aerosol vaccination with NYVAC or MVA in conjunction with a protein subunit vaccine may induce genital immunogen-specific IgA responses as well as further boosting systemic humoral responses.

In the current investigation, we have demonstrated that aerosol-delivered NYVAC- and MVA-based vaccines are safe and immunogenic, inducing long-lasting systemic and mucosal immune responses. Given the advantages of aerosol vaccine delivery, namely speed, simplicity, safety, and cost effectiveness, aerosol vaccination with recombinant poxvirus-based NYVAC and MVA vaccines could offer a viable solution for future mass vaccination campaigns against mucosally transmitted diseases.

Methods

Animals. Animals were captive-bred, mature (4–9 years old, 4- to 9-kg body weight), Indian rhesus macaques (*Macaca mulatta*) from the Biomedical Primate Research Center. Animals were in good health and, for the vaccination study, met the following criteria: no previous immunosuppressive treatment; negative for STLV, SRV, and SIV; low/no IFN γ , IL2, or IL4 responses against HIV env, gag, pol, or nef antigens; cooperative to undergo aerosol immunization. All study protocols were preapproved by the Institutional Animals' Care and Use Committee in accordance with international ethical and scientific guidelines.

Vaccines. DNA-C and NYVAC-C prophylactic HIV Clade C vaccines were developed within the EuroVacc vaccine initiative. The insert immunogens have been previously described (11, 30). MVA-HPV was kindly provided by Transgene (31).

Radiolabeling of Poxviruses and Albumin Macroaggregates. To monitor the biodistribution of the aerosolized poxviruses, 5% of the total vaccine doses, 2.51×10^6 DCI50 NYVAC-C or 5.5×10^6 pfu MVA-HPV, were ^{99}Tc radiolabeled as previously described (32).

Aerosol Administration of Poxviruses. For virus deposition experiments, a cohort of two male and two female rhesus macaque monkeys were used for each poxvirus. Animals were lightly anesthetized with ketamine hydrochloride and pretreated with atropine to reduce salivation during inhalation. Animals were positioned in an adapted isolation chamber to ensure vaccine containment. The reservoir of the nebulizer was loaded with 4 ml of radiolabeled virus preparation, and the airtight mask was fitted over the muzzle of the animal. The nebulizer was especially modified to adjust the minute ventilation airflow volume for each animal during tidal breathing. Aerosol

administration was performed during 3 min by using three 1-min intervals separated by 30-sec pauses to avoid overheating of the vaccine preparations.

Monitoring Biodistribution of Radiolabeled Poxviruses by γ Camera Imaging.

Ten minutes after aerosol administration, the animals were reanesthetized and placed for static scintigraphic examination. Scintigraphic imaging was performed as previously described (32). Subsequently, one animal from each vaccine group was killed immediately after imaging. The sinus, pharynx, trachea, esophagus, lung, and stomach were collected and radioactivity counted by direct scintigraphy. In addition, the following organs were collected and counted for total radioactivity by using both an activimeter and by scintigraphy: eyes, nose skin, superior mandibular, and thyroid.

Histology. Approximately 72 h after aerosol vaccine administration, the three remaining animals from each group (six total) were killed, and organs were collected for histopathological analysis (sinus, pharynx, trachea, lung, eye, brain, liver, kidneys, and ovaries or testes). Histopathologic evaluation was performed by an independent pathologist.

Immunization. Initially, all animals were immunized twice i.m. (weeks 0 and 4) with a DNA HIV clade C vaccine (DNA-C) consisting of an equimolar mixture of plasmids pORT-gp120 and pORT-GPN (final total DNA concentration, 1.05 mg/ml). Animals were injected with 2 ml of DNA-C in each thigh (4.2 mg of DNA-C total).

Subsequently, animals were split into two groups and boosted at weeks 20 and 24 with the attenuated poxvirus NYVAC-C containing the identical HIV immunogens. In Group I, five animals were administered NYVAC-C i.m. in the upper left arm ($10^{7.7}$ pfu in 1 ml). In Group II, 10 animals were administered NYVAC-C ($10^{7.7}$ pfu in 4 ml) to the respiratory tract as an aerosol. To augment the number of animals in the i.m. immunized control group for the CMI arm of the study, results from a parallel vaccination trial were included for analysis. These animals ($n = 10$) were raised in the same facility, immunized i.m. with the same vaccines, with the same vaccine quantities, and by using the same vaccination schedule.

Sample Collection. Blood samples (heparin-treated blood), PBMCs, and mucosal washes (nasal, vaginal, and rectal) were collected as described (33). For the snip biopsies, the animals were sedated, and 5-mm samples were taken.

Th1/Th2 ELISPOT Analysis. Enumeration of HIV-specific IFN γ , IL2, and IL4 cytokine-secreting cells was performed by ELISPOT analysis as previously

described (34). For the stimulation of cytokine production, fresh or cryofrozen PBMCs were cultured with 10 μ g/ml HIV peptides. The HIV peptide antigens consisted of 15-mers with 11-aa overlap spanning the entire clade C of HIV-1 CN54 Gag-Pol-Nef immunogen as well as the gp120 envelope protein (Synpep Corporation) (SI Fig. 9). Reactive spots were quantified by an automatic counter (Aelvis). IFN γ , IL2, and IL4 ELISPOT results were expressed as spot-forming cells (SFC) per 10^6 PBMCs minus background (mean of medium control \pm 2 SD). A positive IFN γ responder was defined as a minimum 50 SFC/ 10^6 PBMCs per peptide pool. Positive IL2 and IL4 responders were defined as 10 SFC/ 10^6 PBMCs per peptide pool.

Antibody Assays by ELISA. ELISAs were established to measure gp120- and NYVAC-specific antibody responses in serum and mucosal secretions. Recombinant CN54 WT gp140 was kindly provided by S. Jeffs (National Institute for Biological Standards and Control, Hertfordshire, U.K.) and the NYVAC antigen was provided by M.E. Immunoplates (HA Nunc) were coated overnight at 4°C with 100 ng per well of HIV-1 clade C recombinant envelope glycoprotein in NaHCO₃ buffer (pH 8.5) or 1 μ g per well of NYVAC antigen in PBS (pH 7.1). After rinsing three times with PBS wash buffer (0.05% Tween 20, PBS), plates were blocked for 2 h at 37°C with PBS (pH 7.1), FCS 10%. Serum samples were diluted 1:10 and mucosal samples were diluted 1:2 in PBS (pH 7.1), FCS 10%, added to wells, and incubated for 120 min at 37°C. After washing, a goat IgG anti-monkey IgA or IgG Fc peroxidase conjugate (Nordic Immunological Laboratories) diluted 1:2,500 was added and the plates were incubated for another 60 min at 37°C. Plates were further washed and incubated in the dark for 30 min at room temperature with 100 μ l per well of OPD substrate. Reactions were stopped with 100 μ l of 1N HCl. Samples were run in duplicate or triplicate depending on sample amounts. Optical density (OD) was measured at 492 nm with an automatic plate reader. Blanks (mean value) were subtracted from the data. Antibody titers were expressed as a ratio of specific to total IgG or IgA amount in each sample.

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